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Ribosome Biogenesis in African Trypanosomes Requires Conserved and Trypanosome-Specific Factors

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Large ribosomal subunit protein L5 is responsible for the stability and trafficking of 5S rRNA to the site of eukaryotic ribosomal assembly. In *Trypanosoma brucei*, in addition to L5, trypanosome-specific proteins P34 and P37 also participate in this process. These two essential proteins form a novel preribosomal particle through interactions with both the ribosomal protein L5 and 5S rRNA. We have generated a procyclic L5 RNA interference cell line and found that L5 itself is a protein essential for trypanosome growth, despite the presence of other 5S rRNA binding proteins. Loss of L5 decreases the levels of all large-subunit rRNAs, 25/28S, 5.8S, and 5S rRNAs, but does not alter small-subunit 18S rRNA. Depletion of L5 specifically reduced the levels of the other large ribosomal proteins, L3 and L11, whereas the steady-state levels of the mRNA for these proteins were increased. L5-knockdown cells showed an increase in the 40S ribosomal subunit and a loss of the 60S ribosomal subunits, 80S monosomes, and polysomes. In addition, L5 was involved in the processing and maturation of precursor rRNAs. Analysis of polysomal fractions revealed that unprocessed rRNA intermediates accumulate in the ribosome when L5 is depleted. Although we previously found that the loss of P34 and P37 does not result in a change in the levels of L5, the loss of L5 resulted in an increase of P34 and P37 proteins, suggesting the presence of a compensatory feedback loop. This study demonstrates that ribosomal protein L5 has conserved functions, in addition to nonconserved trypanosome-specific features, which could be targeted for drug intervention.

Biosynthesis of proteins is an essential process in all living cells. Ribosomes, large ribonucleoprotein complexes, are responsible for this process across all domains of life. They are composed of two subunits. In eukaryotes, the 40S small subunit (SSU) contains a single 18S rRNA and more than 30 proteins, while the 60S large subunit (LSU) consists of three rRNA molecules (25/28S, 5.8S, and 5S rRNAs) as well as more than 40 proteins (1). While the rRNAs perform the catalytic function of protein synthesis, the protein components provide the structural scaffold of the ribosome and contribute to shaping interaction sites with accessory factors (2–4). In addition to ribosomal proteins, more than 170 accessory proteins are involved in the ribosomal biogenesis pathway. These proteins participate in the maturation, trimming, and modification of the rRNAs, the transport of the precursors across different cellular compartments, and the assembly of a translation-competent ribosome (5). The pathway of ribosome biogenesis is evolutionarily conserved throughout the eukaryotic phylogenetic tree. Early stages of ribosomal biogenesis take place in the nucleolus, where all of the rRNAs except 5S rRNA are transcribed as a single large primary transcript by RNA polymerase I and are processed into 5.8S, 18S, and 25/28S rRNAs (6). In eukaryotes, 5S rRNA is a small and essential rRNA of 120 nucleotides whose function in protein synthesis is thought to be mediated by its many contacts with functionally important sites on the 25/28S rRNA (7, 8). The 5S rRNA is usually transcribed independently by RNA polymerase III in the nucleoplasm (7), with the 5S rRNA from the yeast *Saccharomyces cerevisiae* being an exception to this rule. Large ribosomal protein L5 binds 5S rRNA in the nucleoplasm after its processing and maturation. This binding plays an important role in both stabilizing and trafficking 5S rRNA to the nucleolus for ribosomal assembly (9–11).

Although most aspects of ribosome biogenesis are conserved in eukaryotes, a few unique characteristics have been described in the early-diverging eukaryote *Trypanosoma brucei*. This organism is a single-cell parasite responsible for human African sleeping sickness and nagana in cattle. One of the striking differences is the processing of rRNA, where one of the LSU rRNAs (the 25/28S rRNA) is further fragmented into six matured transcripts (12, 13). In addition, the recently published structure of the mature *T. brucei* ribosome has revealed the existence of large protruding expansion segments in both the 25/28S rRNA and the 18S rRNA (14). Sequence comparisons of ribosomal proteins from *T. brucei* with those from other eukaryotes showed large *T. brucei*-specific protein extensions. Structural analysis of these ribosomal proteins shows that specific helical extensions interact with the additional rRNA sequences (14). Our laboratory has also identified trypanosome-specific ribosome biogenesis proteins P34 and P37 (15). These two nearly identical RNA binding proteins are essential for ribosomal biogenesis and survival of the parasite (16). In other organisms, 5S rRNA stability and translocation from the nucleoplasm to the nucleolus are mediated by L5, whereas in *T. brucei*, P34 and P37 are also required for these processes (17). Loss of these two proteins causes decreases in 5S rRNA levels and 80S ribosome formation and an overall decrease in protein synthesis (16). These phenotypes are similar to those observed in yeast L5-knockout cell lines (10). In addition, our laboratory has previously reported on the sequence divergence of L5 at key 5S rRNA binding positions which have been shown *in vitro* to result in lower 5S rRNA binding activity (17). These data, combined with the existence of novel 5S rRNA binding factors, have prompted us to take a more detailed look at the functions of L5 in *T. brucei*. Here we have used RNA interference (RNAi) to decrease the expression of

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TABLE 1 Oligonucleotides used in this study

Name	Expt	Oligonucleotide sequence (5′→3′)	Reference or source
L5 RNAi BamHI For	Cloning	GCGGATCCGTTGGCGACGAGGTTG	This study
L5 RNAi XhoI Rev	Cloning	GCTCGACTTTGAGCTTGCCTCCTC	This study
L5 qPCR For	Real-time PCR	TGAGGCGAAACCGAAGAAGT	This study
L5 qPCR Rev	Real-time PCR	ACAGCGGCAACCTTTGCTTT	This study
TERT qPCR For	Real-time PCR	GAGCGTGTGACTTCCGAAGG	19
TERT qPCR Rev	Real-time PCR	AGGAACTGTCACGGAGTTTGC	19
5S	Northern blotting	GCATTCGGCCAAGTATGGTC	27
28S (LSU1)	Northern blotting	GTCCTGCCACACTCAGGTCTGA	27
18S	Northern blotting	AAATGATCCAGCTGCAGGTTCACCTA	27
ITS2	Northern blotting	ATCACTCACTACACACACGTAT	This study
ITS3	Northern blotting	ACGACAATCACTCACACACACATGGC	52
ITS7	Northern blotting	TATGTAGTACCACACAGTGTGACGCG	52
Junction between ITS2 and 5.8S	Northern blotting	TTGTTTTTATATTCGACACTGAGAA	52
Pre-18S	Northern blotting	TCAAGTGTAAGCGCGTGATCCGCTGTGG	49
7SL	Northern blotting	CAACACCGACACGCAACC	This study
L5 For	Northern blotting	ACTACAAGCGGTTCCAGGTG	This study
L5 Rev	Northern blotting	GGACGATCTGGGCAATCAC	This study
L3 For	Northern blotting	GGCGCTTCTACAAGAACTGG	This study
L3 Rev	Northern blotting	ACCGTAGCCAACAAAACCAC	This study
L11 For	Northern blotting	TTAACATTTGCGTGGGTGAA	This study
L11 Rev	Northern blotting	TTAACATTTGCGTGGGTGAA	This study
S5 For	Northern blotting	CTCCAGACGACGGAGATAGC	This study
S5 Rev	Northern blotting	ATCGGTGACACATCAACAGC	This study
Tubulin For	Northern blotting	ATGCGTGAGGCTATCTGCATCCACATTGGT	This study
Tubulin Rev	Northern blotting	CTCAATATCGAGGTTGCGGCGAGTCAAAT	This study

L5 and analyzed the resulting cell line to delineate the involvement of L5 at different steps of the ribosomal biogenesis pathway.

MATERIALS AND METHODS

Cell growth, transfection, and generation of the L5-knockdown cell line. Procyclic-form *T. brucei* strain 29-13, which expresses both T7 RNA polymerase and the tetracycline (Tet) repressor for Tet-regulated expression of introduced genes, was grown in Cunningham's medium supplemented with 10% fetal bovine serum (16). The medium also contained 15 μg/ml of G418 and 50 μg/ml of hygromycin to maintain the T7 RNA polymerase and Tet repressor constructs. The Tet-inducible L5 RNAi plasmid p2T7177-L5 was constructed by insertion of a 464-nucleotide fragment (encompassing nucleotides 209 to 672) from the coding region of L5 (TryTripDB accession number Tb 427tmp.244.2740) between two head-to-head T7 promoters under the control of tetracycline operators. Primers for this construct are listed in Table 1. NotI-linearized p2T7177–L5 was transfected by electroporation (Amaxa Nucleofactor II) into procyclic 29-13 cells. Cells expressing this construct were selected with 2.5 μg/ml phleomycin, and clonal cell lines were generated by limiting dilution (16). Cells were induced with 2.5 μg/ml Tet for L5 double-stranded RNA (dsRNA) expression, and growth curves for wild-type, uninduced, and Tet-induced L5 RNAi cells were determined as the products of cell density and total dilution. All growth curves were performed in triplicate.

qRT-PCR. Total RNA was isolated from wild-type, uninduced, and Tet-induced L5 RNAi cells using the TRIzol reagent according to the manufacturer's instructions (Life Technologies). Isolated RNA was treated with DNase I (Turbo DNA-free kit; Ambion); cDNA was prepared using random hexamers (Applied Biosystems) and SuperScript III first-stand synthesis Supermix (Life Technologies) per the manufacturer's recommendations. Quantitative reverse transcription-PCR (qRT-PCR) was performed, and the results were analyzed as previously described (18) using IQ SYBR green Supermix and the primers listed in Table 1. cDNA starting quantities were normalized to the quantity of telomerase reverse transcriptase (TERT) (19). Real-time PCR was performed on three biological replicates. Values are expressed as means ± standard deviations.

Northern analysis. RNA was extracted as described for qRT-PCR. Five micrograms of total RNA was electrophoresed on 1% formaldehyde agarose gels and transferred to Nytran SuPerCharge membranes (Whatman) by capillary transfer and subsequently cross-linked by UV illumination (UV Stratalinker 2400; Stratagene). To detect rRNAs and their precursors, oligonucleotide probes radiolabeled with γ-^{32}P at their 5′ ends (Table 1) were used to probe membranes in ULTRAhyb hybridization buffer (Ambion) at 42°C overnight and washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS twice at room temperature and once at 42°C. For mRNA detection, gene-specific primers (Table 1) were designed, and amplified PCR products were randomly labeled with [α-^{32}P]dATP using a RadPrime DNA labeling system (Life Technologies), followed by hybridization as described above. Signals were quantified using a phosphorimager (Bio-Rad) with Quantity One software. All Northern analyses were performed on three biological replicates, and representative results are shown. Values are reported as means ± standard deviations.

Western analysis. Whole-cell protein extracts were prepared as previously described (20). Proteins were separated by electrophoresis in a 12% SDS-polyacrylamide gel. Western blot analysis was then performed using antibodies directed against L5 (17), P34/P37 (17), L3 and L11 (Thermo Scientific), and S5 (Novus Biologicals) using dilutions of 1:2,000, 1:2,000, 1:500, 1:500, and 1:500, respectively. Antibodies directed against elongation factor 2 (EF-2; 1:200 dilution; Santa Cruz) were used as a loading control. Densitometric analysis was performed using a Chemidoc gel imaging system (Bio-Rad) in combination with Image Lab software. All Western analyses were performed on at least three biological replicates, and representative results are shown. Values are reported as means ± standard deviations.

Polysome analysis. Ribosomal subunits, monosomes, and polysomes were separated by velocity sedimentation on 10 to 40% sucrose gradients and analyzed by monitoring the UV absorbance at 260 nm along the

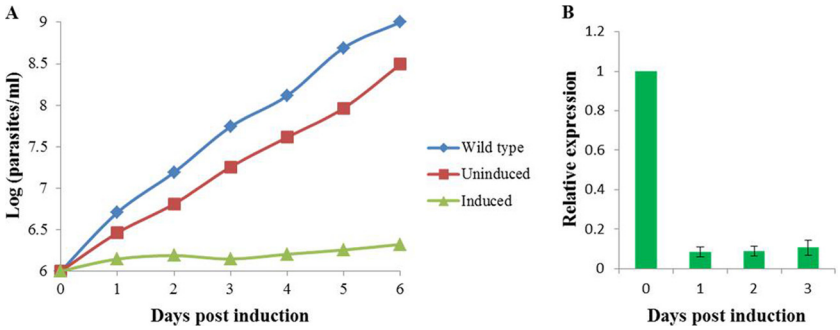

FIG 1 L5 is essential for procyclic *T. brucei* cells. (A) Parasites transfected with vector p2T7177–L5 were grown in the presence of tetracycline to induce expression of dsRNA. The growth of uninduced cells was compared to that of induced RNAi cells and wild-type untransfected cells. (B) The extent of L5 knockdown was verified by quantitative RT-PCR. A value of 1 corresponds to unchanged transcript levels relative to those at day 0 postinduction. Lower values indicate transcript depletion. Error bars are used to indicate standard deviations of triplicate experiments.

gradient as described previously (16). For both wild-type and induced cell samples, a total of 1×10^9 cells were used per gradient. Induced samples were collected at day 2 postinduction in order to avoid nonspecific effects of RNAi. For fraction collection, the peak separation function at setting 3 of an Isco UA-6 UV detector was used in conjunction with an Isco Retriever 500 fraction collector. Four biological replicates of wild-type and induced polysomal profiles were performed to ensure consistent results. Equal volumes of fractions were collected from both wild-type and induced samples for further processing. RNA was purified from the collected fractions using the TRIzol LS reagent (Life Technologies) at a ratio of 3:1 (vol/vol, TRIzol/sample). Probes directed against the 25/28S rRNA, the 18S rRNA, and the 5S rRNA (for mature rRNA species) and probes directed against internal transcribed spacer 7 (ITS7) and the junction between 5.8S rRNA and ITS2 (for unprocessed precursors) were used in Northern blot analysis of the gradient RNA fractions (the probes are listed in Table 1). Northern analyses from fractions of the polysomal profiles were performed on two biological replicates. Proteins from the gradient fractions were recovered by methanol-chloroform precipitation and analyzed by Western blotting using antibodies directed against L5, P34/P37, and EF-2 (dilutions, 1:500, 1:500, and 1:100, respectively). Western analyses were performed on three biological replicates, and representative blots are shown.

Subcellular fractionation. Cytoplasmic and nuclear extracts were prepared as described previously (21), with some modifications. For lysis, cells were passed through a 25-gauge needle 3 times and sonified using 10 s on/30 s off cycles for a total of 3 min of on time at setting 2 on a 550 Sonic Dismembrator homogenizer (Fisher Scientific). The crude nuclear extracts were also passed through a 21-gauge needle 3 times and sonified at setting 2.5 using 10 s on/30 s off for a total of 2 min of on time. Protein-RNA complexes were recovered from the nuclear and cytosolic extracts using methanol precipitation as described previously (22) for subsequent Western and Northern blot analyses. Western blot analyses were performed using antibodies directed against the cytosolic marker EF-2 and the nuclear marker TATA-binding protein (TBP) (23). RNA was purified from a methanol-precipitated extract using the TRIzol LS reagent. Probes directed against 18S rRNA, ITS7, and the junction between 5.8S rRNA and ITS2 were used in the Northern blot analyses (Table 1).

Immunoprecipitation. Immunoprecipitations were performed as previously described (24). Protein A-labeled magnetic beads (Dynabeads, Life Technologies) were blocked in phosphate-buffered saline (PBS)–bovine serum albumin and incubated with anti-P34/P37 or anti-L5 antibody for 1 h at 4°C. The antibody was chemically cross-linked to the beads using dimethyl pimelimidate (DMP; Thermo Scientific, Pierce). The cross-linking reaction was quenched with 50 mM ethanolamine (Sigma-Aldrich) in PBS, and excess antibody was eluted in two successive washes with 1 M glycine, pH 3. Beads coated with anti-P34/P37 or anti-L5 antibody were washed in PBS-Tween 20 (PBS-T) and incubated with 100 μg whole-cell extract at 4°C for 1 h. The supernatant was removed, the beads coated with antibody-antigen complexes were washed in PBS-T three times, and the protein complexes were eluted from the beads by resuspension in SDS-PAGE sample buffer, followed by incubation at 70°C for 10 min. The final eluted fractions and the supernatants were analyzed by SDS-PAGE, followed by Western blotting using the anti-L5 or anti-P34/P37 antibody. The experiments were performed on three biological replicates, and representative blots are shown.

RESULTS

Ribosomal protein L5 is essential for *T. brucei*. As the major 5S rRNA binding protein, L5 is essential in yeast (10, 25) and *Escherichia coli* (26), the two organisms in which the essentiality of L5 has been studied. Trypanosomes express novel 5S rRNA binding proteins P34 and P37 (27), and the sequence of L5 deviates from the eukaryotic L5 consensus sequence at potentially significant positions (17). Therefore, we wished to determine whether L5 is an essential factor in *T. brucei*. We generated a procyclic cell line containing a p2T7-derived construct (p2T7177–L5) which can drive the transcription of an L5 dsRNA in the presence of tetracycline. Appropriate insertion of the vector (which targets the endogenous 177-bp repeat locus) in the transfectant cell line was confirmed by PCR (data not shown).

We then analyzed the growth of this cell line in the absence or presence of an inducer (Fig. 1A). When the cells were allowed to grow in the absence of tetracycline (i.e., the cells expressed L5), the growth curve (Fig. 1A, red closed squares) was similar to that of wild-type procyclic cells (Fig. 1A, blue diamonds). However, following induction of dsRNA expression with tetracycline, cell growth was completely halted through at least day 5 postinduction (Fig. 1A, green triangles). In these cells, the levels of L5 mRNA were decreased by 91.4% ± 2.5% on day 1 postinduction and remained depleted at least through day 3, as verified by quantitative RT-PCR (Fig. 1B). L5 protein levels decreased by 74.6% ± 4% by the third day of induction (Fig. 2B). Uninduced cells grew slightly more slowly than wild-type cells, likely due to a low level of expression of the RNAi even in the uninduced cells (i.e., the leakiness of the construct). Indeed, the levels of L5 mRNA in uninduced cells were decreased 4.8% relative to those in the parental wild-type cells (data not shown). These results demonstrate that L5 is an essential protein in procyclic *T. brucei*, despite the presence of novel and essential 5S rRNA binding proteins P34 and P37.

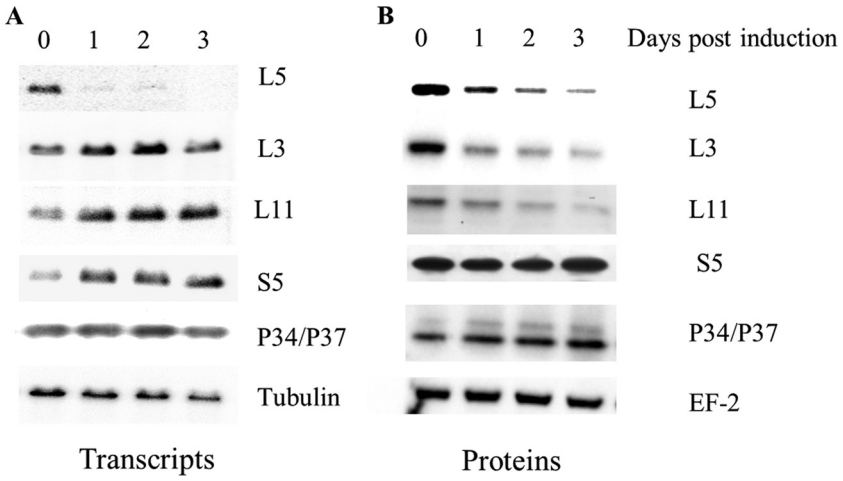

FIG 2 Loss of L5 leads to a specific decrease in LSU protein levels without a decrease in their mRNA levels and to an increase in P34 and P37 protein levels. (A) Northern blot analysis was performed on whole-cell RNA after induction of the L5 dsRNA using probes for the genes indicated on the right side of the blot. Uninduced day 0 samples were compared to induced samples for three consecutive days, days 1 to 3. A probe against the tubulin gene was used as a loading control. (B) Whole-cell protein samples were taken at the same time points described for panel A and were subjected to Western blot analysis. An antibody against EF-2 served as a loading control.

In the absence of L5, the levels of ribosomal protein transcripts are increased but the levels of large-subunit proteins are specifically decreased. First, we wanted to determine whether there were changes in the levels of steady-state transcripts resulting from the loss of L5 in trypanosomes. Total RNA was isolated from procyclic cells after induction of RNAi, and Northern blot analysis was performed using probes for the transcripts of L5, L3, L11, S5, P34/P37, and tubulin. Immediately upon induction of the dsRNA, the mRNA levels of L5 decreased sharply (Fig. 2A). This decrease was specific to L5, as demonstrated by the unchanged levels of tubulin mRNA. Interestingly, the steady-state levels of the mRNAs for other ribosomal proteins, namely, L3, L11, and S5, actually increased at least 2-fold (2.1 ± 0.1-fold for L3, 2.8 ± 0.2-fold for L11, and 2.4 ± 0.2-fold for S5 at day 2) in the induced samples, on the basis of the findings of an average of 3 experiments. No major alterations in the levels of P34/P37 mRNA were observed.

We next determined the effects of the loss of L5 on the steady-state protein levels of a set of ribosomal and ribosome-related proteins. Cell extracts were prepared on each day after induction of the L5 dsRNA, and Western blot analysis was performed using antibodies raised against large ribosomal subunit proteins L5, L3, and L11, small ribosomal subunit protein S5, the trypanosome-specific P34 and P37 involved in ribosomal biogenesis, and a control antibody against EF-2. Among the ribosomal proteins, L5, L3, and S5 were incorporated in the 90S particle, the earliest step of the biogenesis pathway, whereas L11 was a late associating protein and was part of the 66S particle. As shown in Fig. 2B, induction of the RNAi cell line with tetracycline led to an expected decrease in the steady-state levels of the L5 protein of 74.6% ± 4% by day 3 postinduction. In contrast to the steady-state levels of their transcripts, the ribosomal proteins L11 and L3 exhibited concomitant decreases of 76.7% ± 3% and 81.2% ± 2%, respectively, by day 3 postinduction, suggesting the existence of feedback mechanisms that maintain the stoichiometry of the large ribosomal subunit proteins. The decrease in protein levels was specific to the large subunit, since the levels of S5 remained unchanged. However, after induction the levels of P34 and P37 were slightly but consistently increased by 21% ± 1.2% on day 3. This raises the possibility that *T. brucei* cells respond to a decrease in one 5S rRNA binding factor by increasing expression of a distinct set of unique 5S rRNA binding proteins. The expression of the control EF-2 was not altered in the induced cells.

In the absence of L5, the steady-state levels of the large-subunit rRNA are specifically decreased. The levels of ribosomal proteins are often coupled to the levels of rRNAs (28). In order to address the possibility that similar mechanisms exist in *T. brucei*, we performed Northern blot analysis using RNA extracted from our L5 RNAi cell line at increasing times after induction. We used probes specific for 5S rRNA, 25/28S rRNA (LSU rRNA), and 18S rRNA (SSU rRNA). The results showed that upon induction of the L5 dsRNA, the steady-state levels of 5S rRNA fell rapidly to 58.2% ± 3% at day 1 (Fig. 3). Our laboratory has previously shown that *T. brucei* L5 binds directly to 5S rRNA, so our finding that L5 is also necessary for 5S rRNA stability is consistent with its role as a 5S rRNA binding protein. Additionally, we investigated whether 25/28S rRNA (which is also present in the large subunit) was affected by the L5 knockdown. Our results showed that the levels of 28S rRNA (detected by the LSU1 probe) were also significantly decreased to 31.2% ± 2% at day 3 upon induction with

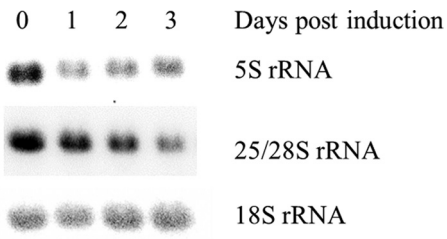

FIG 3 Loss of L5 leads to a specific decrease in the levels of large-subunit rRNAs. RNA extracted from cells at days 0 to 3 after induction of the L5 dsRNA was analyzed by Northern blotting using probes directed against LSU1 of the 25/28S rRNA, large-subunit 5S rRNA, and small-subunit 18S rRNA.

tetracycline (Fig. 3). Interestingly, the kinetics of destabilization for 28S rRNA were slower than that in the case of 5S rRNA, suggesting a more immediate effect on 5S rRNA stability and a delayed and perhaps indirect effect on 28S rRNA. Finally, we analyzed the steady-state level of the small-subunit 18S rRNA and found that it was not affected after induction of L5 RNAi (Fig. 3, bottom). This supports the hypothesis that the effects of depleting L5 are specific to the large ribosomal subunit.

Processing of rRNAs in the absence of L5 is abnormal, leading to accumulation of precursors. rRNA processing and maturation are complex processes that occur primarily in the nucleolus. They consist of a series of specific cleavages of a large precursor and several intermediates to generate mature rRNA (29). In trypanosomes, an initial cleavage generates a precursor to the 18S rRNA and a precursor to the 5.8S and 25/28S rRNAs (Fig. 4A). Another endonucleolytic cleavage separates the 5.8S rRNA precursor from the LSU rRNA precursor. The latter is further cleaved into six segments (LSU1, LSU2, small RNA 1 [SR1], SR2, SR4, and SR6) (12, 13, 30). Because our results indicated defects in the production of mature large-subunit rRNAs, we examined the maturation pathway of these species.

We used Northern blot analysis and selected probes that would specifically detect intermediates that might accumulate if processing was defective. Probe ITS2 hybridizes to a region that lies between the 5.8S rRNA and the LSU rRNA and allows detection of the 9.2-kb precursor as well as the 5.8-kb and the 0.61-kb species depicted in Fig. 4A. Our results showed that, upon induction of the dsRNA for L5, there was an accumulation of the 5.8- and 0.61-kb species detectable with probe ITS2 (Fig. 4B). Another probe directed against a region internal to the LSU precursor, ITS3, detects the intermediates of 5.8 kb, 5.0 kb, and 3.9 kb (Fig. 4A). All of these species accumulated in the absence of L5 (Fig. 4B). Probe ITS7 is directed against a region further downstream and does not recognize the 3.9-kb intermediate but hybridizes with the 5.8-kb and the 5.0-kb intermediates. Both of these products accumulated (2.5 ± 0.4-fold for the 5.8-kb species and 4.6 ± 0.9-fold for the 5.0-kb species on day 1) after induction of L5 RNAi and were detected with the ITS7 probe (Fig. 4B). In addition, a probe directed against the junction region between 5.8S and ITS2 was designed to identify both intermediates recognized by the ITS2 probe and the mature 5.8S rRNA (also a component of the large subunit). Our results utilizing this probe revealed that not only does the 0.61-kb species accumulate (5.7 ± 0.9-fold on day 1) but also there is a decrease in the level of mature 5.8S rRNA (28.8% ± 1.2%). To detect whether L5 plays a role in the maturation of the small-subunit rRNA, we used a probe directed against the pre-18S rRNA species designed not to detect the mature 18S rRNA. This probe can hybridize with the 9.2-kb precursor and with 3.4-kb intermediates. We found that both these species accumulate (2.8 ± 0.6-fold for the 9.2-kb species and 2.1 ± 0.5-fold for the 3.4-kb species on day 1) in the induced sample (Fig. 4B), indicating an involvement of L5 in the processing of the 18S rRNA. By the third day of the induction, the observed accumulation of rRNA precursors was somewhat diminished, probably as a result of nonspecific effects of RNAi, since L5 protein levels remained low (Fig. 2B). As a loading control in these experiments, we used a probe specific for the 7SL RNA (Fig. 4B). Taken together, our data implicate L5 in the maturation of *T. brucei* rRNA precursors.

L5 is essential for ribosomal biogenesis in *T. brucei*. Ribosomal precursors are not normally incorporated into functional ribosomes, so we wished to directly assess whether overall ribosome assembly was affected in our L5-knockdown cell line. We treated cells with cycloheximide to stop translation while maintaining polysomes intact and then separated them on sucrose gradients using velocity sedimentation (16). The profile (A_{260}) across the gradients in the wild-type cell line (Fig. 5A, left) reveals the positions of the ribosomal subunits, the mature 80S ribosomes, and polysomes containing increasing numbers of ribosomes bound to mRNAs. However, when we analyzed extracts obtained from induced cell lines (Fig. 5A, right), we observed a diminished peak for the 60S ribosome, as well as a significant decrease in the 80S peak. These results indicate that these cells are not able to assemble functional large ribosomal subunits. Alternatively, the rate of dissociation of properly assembled large subunits could be higher in cells lacking L5. The accumulation of 40S subunits, as indicated by the larger 40S peak in Fig. 5A (right), further confirms that in the absence of L5, the defects in the assembly of the large subunit lead to an excess of small subunits unable to assemble with the 60S subunit to form a mature ribosome. Additionally, we observed a marked decrease in the polysomal peaks. This further indicates that proper ribosomal assembly is impeded in the absence of L5.

To confirm the identity of the peaks, Northern analysis was performed on fractions from both wild-type- and RNAi-induced samples using probes directed against 25/28S rRNA and 5S rRNA (constituents of the 60S subunit) and against 18S rRNA (a constituent of the 40S subunit) (Fig. 5B). As expected, 25/28S and 5S rRNAs were detected in the fractions identified as the 60S, 80S, and polysomal peaks along the gradients, and 18S rRNA was detected in fractions corresponding to the 40S, 80S, and polysomal peaks. 5S rRNA was also detected in low-molecular-mass fractions (the low-density region of the gradient toward the left of Fig. 5B). This pool of 5S rRNA likely represents preribosomal 5S rRNA associated with P34, P37, and L5 as well as the free form of 5S rRNA.

Our analysis of whole-cell rRNAs had indicated that intermediate RNA species accumulate in cells lacking L5. We wanted to further determine whether these intermediate RNAs are incorporated into the assembly-deficient ribosomes observed in the polysomal profiles. Using probes directed against ITS7 and the junction region between 5.8S rRNA and ITS2 (which identify 5.8-kb and 0.61-kb molecules, respectively), we performed Northern blot analysis on the gradient fractions from wild-type and induced cells (Fig. 5B). As expected, the intermediates were not detected in the fractions from wild-type cells. However, when the L5 dsRNA was induced and L5 was depleted, the intermediates could be readily detected in the fractions corresponding to the 40S peak and the monosomal peak and (albeit with less intensity) in the polysomal fractions. The fact that these ribosomal subunits contain partially processed rRNAs may further explain why they are incompetent for proper assembly.

To substantiate the argument that in the absence of L5 unprocessed ribosomal species are being exported to the cytoplasm and accumulating in ribosomes, we performed biochemical subcellular fractionation to obtain cytosolic and nuclear fractions. In order to assess the quality of the fractionation, we performed Western blot analysis with antibodies against cytosolic (EF-2) and nuclear (TBP) markers. As shown in Fig. 6A, both markers can be detected in whole-cell extracts of both wild-type and RNAi cell lines. Cyto-

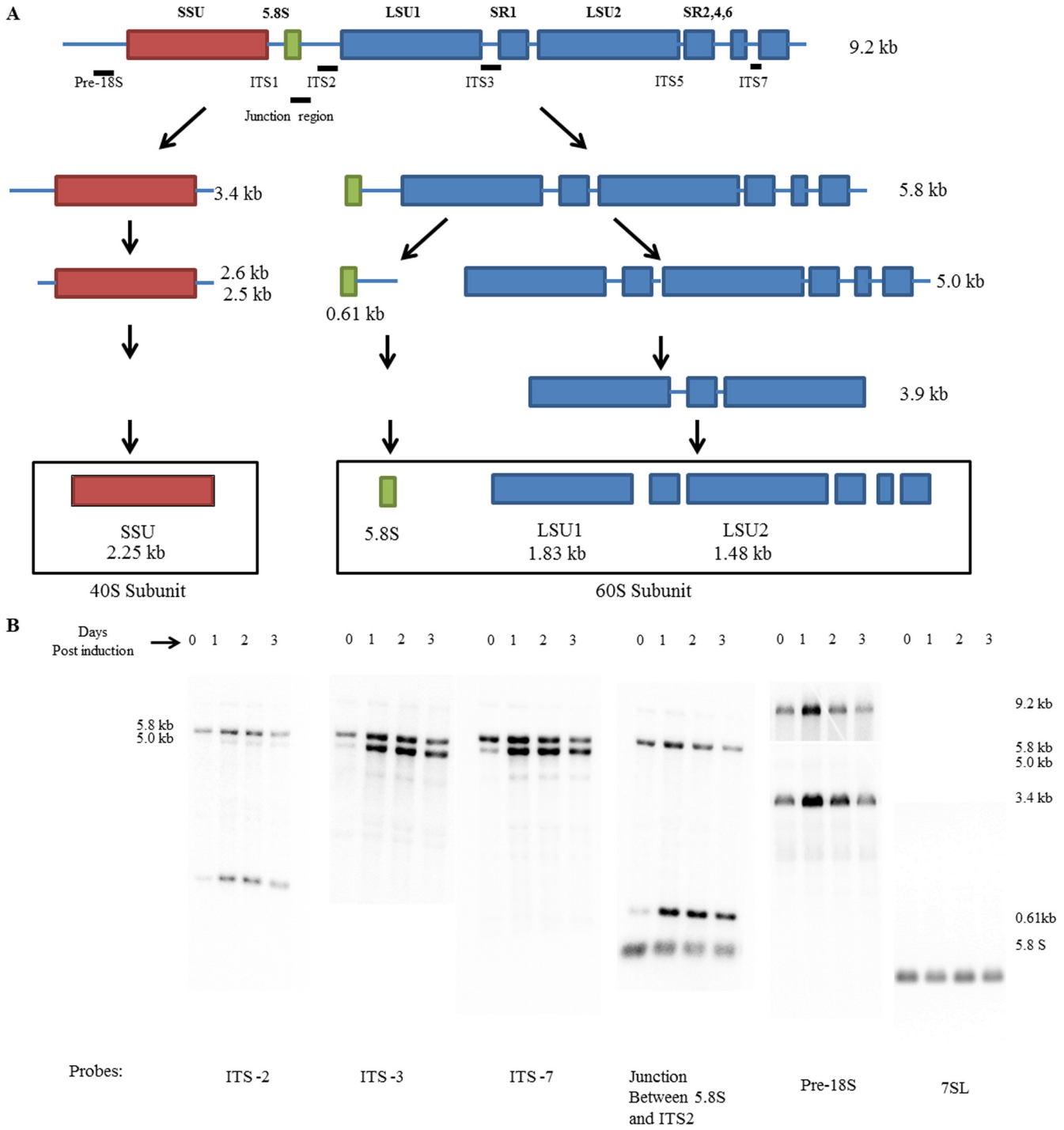

FIG 4 Loss of L5 causes an accumulation of ribosomal precursors. (A) Schematic of maturation steps leading to rRNAs. Red, SSU; green, 5.8S rRNA; blue, various fragments comprising the LSU rRNA (SR, small RNA); arrows, processing events; ITS, internal transcribed spacers; black bars, the positions of the probes used in this study. (B) Northern blot analysis of RNA purified from procyclic cells bearing the L5 RNAi construct at various times postinduction. Probes were selected to identify intermediates in the maturation pathway. For the pre-18S rRNA probe, two different exposures were used to detect the 9.2-kb and the 3.4-kb species.

solic fractions of both cell lines showed a substantial enrichment of EF-2 over TBP, which was barely detectable. Conversely, the nuclear fractions exhibited a substantial enrichment of TBP over EF-2. These results confirmed the quality of the fractionation. We then proceeded to analyze these fractions for the presence of the 5.8-, 5.0-, and 0.61-kb intermediates by Northern blotting in both wild-type and L5-depleted cells (Fig. 6B). As expected, in wild-type cells, rRNA processing intermediates could be detected at low levels in the whole-cell extract and in the nucleus-enriched fractions (Fig. 6B, left and right sets of lanes) but not in the cytosol

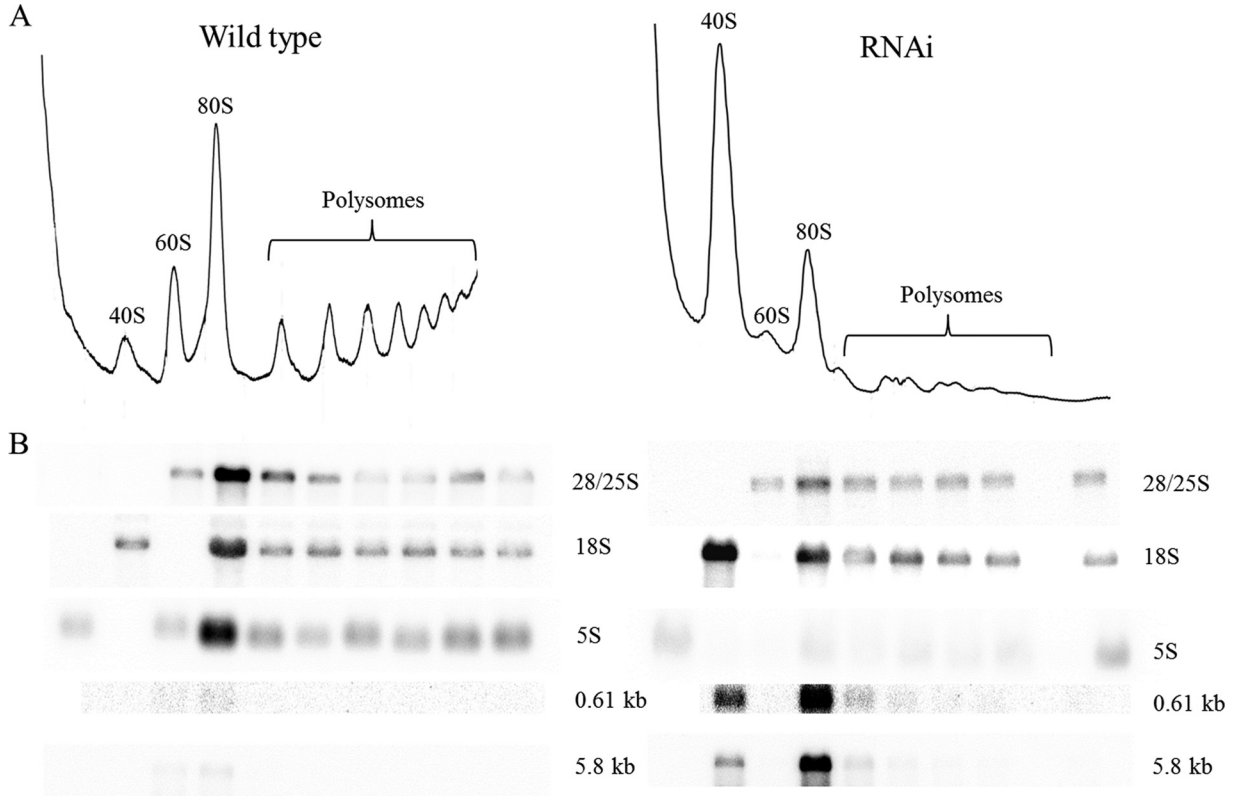

FIG 5 Loss of L5 leads to defects in ribosomal assembly. (A) Polysomal extracts were prepared from wild-type and induced RNAi cells. The extracts were subjected to velocity sedimentation on 10 to 40% sucrose gradients. (B) Northern blot analysis of fractions collected from the polysomal gradients. Wild-type fractions (left) were compared to induced L5 RNAi cells (right). The blots were analyzed using probes directed against 25/28S rRNA, 18S rRNA, 5S rRNA, and the junction region to detect 5.8- and 0.61-kb rRNA intermediates.

(Fig. 6B, middle set of lanes). However, in the absence of L5, these intermediates were found at higher levels not only in the nuclear fraction but also in the cytoplasmic fraction. As a loading control, we probed for 18S rRNA.

Analysis of the gradient fractions by Western blotting (Fig. 7) showed that the L5 protein could be detected in the large ribosome (60S subunit), monosomes (80S), and polysomes, as expected in the wild-type cells. As seen in the analysis of the whole-cell extract, the levels of EF-2 were essentially unchanged in the induced RNAi cells. Upon induction, the levels of L5 were drastically reduced. Interestingly, we observed a 29% ± 4.2% increase in the levels of P34/P37 proteins in the polysomal fractions (Fig. 7, lanes indicated with brackets) relative to the internal standard EF-2 upon induction of the L5 dsRNA. Once again, this may suggest a compensatory mechanism attempting to stabilize 5S rRNA.

Upon depletion of L5, upregulated P34/P37 is specifically recruited to sustain the levels of the P34/P37–L5 complex. We next wanted to evaluate if the increase of P34/P37 levels seen upon depletion of L5 are indeed compensatory. Since the trimolecular complex P34/P37–L5-5S rRNA is essential for 5S rRNA stability and transport, we reasoned that the upregulation of P34/P37 may lead to a mass-action effect that drives the formation of the P34/P37–L5 complex even at lower levels of L5. Therefore, we investigated what percentage of total L5 was bound by P34/P37 under wild-type conditions and under conditions of L5 depletion. We used immune-capture experiments using the anti-P34/P37 antibody to isolate complexes containing P34/P37 from both wild-type and induced L5 RNAi cells (at 2 days postinduction). The pellets and the supernatants were analyzed for the presence of L5 by Western blotting, and the results were quantified and normalized to the levels of L5 in the input (no-immune-capture) reaction. As shown in Fig. 8 (top), we were able to recover 19.5% ± 3% of L5 in the immune-capture pellet (P) under wild-type conditions. However, when L5 was depleted, a larger percentage of the remaining L5 protein was associated with P34/P37 in the immune-capture reaction (43.2% ± 8%). The results for the control consisting of beads alone showed that L5 does not interact nonspecifically with the beads. The complementary experiment reversing the capture and detection antibodies confirmed that in the L5 RNAi cells, P34 and P37 can still be immune captured using beads conjugated to the L5 antibody (Fig. 8, bottom). This experiment suggests that the increase in expression of P34/P37 leads to a mass-action effect that attempts to raise the concentration of the P34/P37–L5 complex to keep it from falling as L5 is depleted.

DISCUSSION

Whereas the enzymatic activity of the ribosome is performed by its RNA components, ribosomal proteins are critical for the structure and function of the ribosome. One of the proteins of the large ribosomal subunit, L5, associates tightly with 5S rRNA to form the central protuberance of the ribosome (31). Unlike other ribosomal proteins, L5 binds 5S rRNA in the nucleoplasm prior to their incorporation into the nascent ribosomal particle. This association, which continues as the particle transits toward the nu-

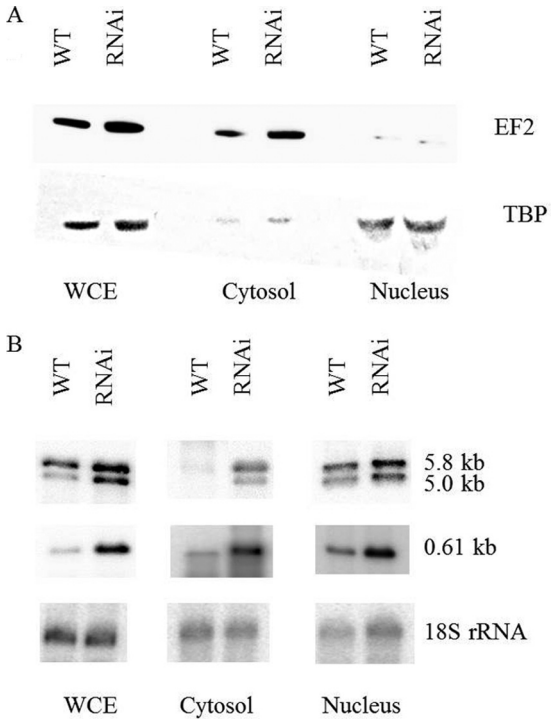

FIG 6 Loss of L5 leads to nuclear export of rRNA processing intermediates. (A) Biochemical fractionation was performed on wild-type and L5 RNAi cell lines to separate nuclear and cytosolic fractions. Antibodies against cytosolic marker EF-2 and nuclear marker TBP were used to confirm the quality of the fractionation. (B) The fractions from panel A were analyzed for the presence of the 5.8-kb, 5.0-kb, and 0.61-kb rRNA processing intermediates. 18S rRNA was used as a loading control. WCE, whole-cell extract.

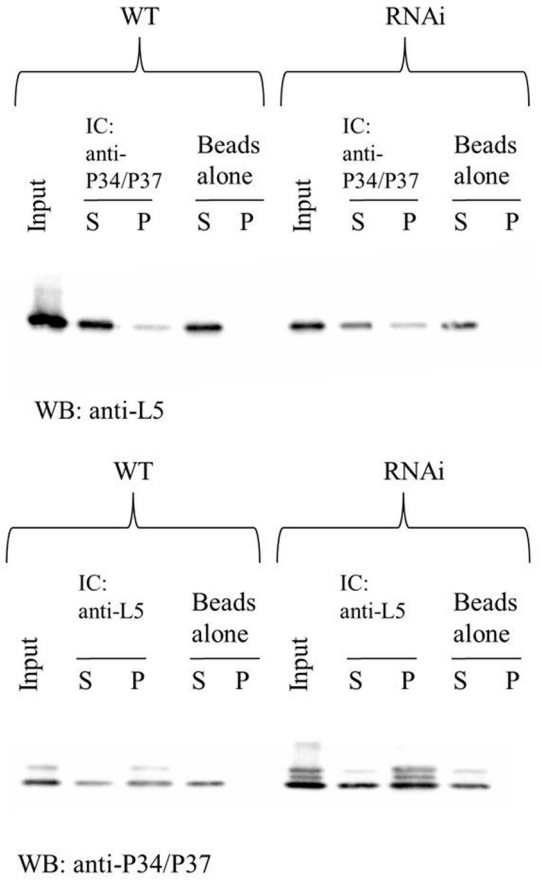

FIG 8 A greater fraction of L5 associates with P34/P37 in induced L5 RNAi cells. (Top) Extracts from wild-type cells (left) and Tet-induced RNAi cells (right) were used in immune-capture (IC) assays with an antibody against P34/P37 (top). The complexes were analyzed by Western blotting (WB) using an L5 antibody. (Bottom) The complementary assay was performed using the L5 antibody conjugated to beads for immune capture and the P34/P37 antibody for Western blotting analysis. In both assays, beads alone were used as controls. S, supernatant; P, pellet.

cleolus, the site of ribosomal biogenesis, serves to stabilize 5S rRNA and protect it from degradation by nucleases (9–11). 5S rRNA is an essential component of the eukaryotic ribosome, so it would be expected that in the absence of a stabilizing factor for 5S rRNA, cells would not be viable. In yeast, *in vivo* depletion of L5 is lethal and leads to defects in LSU rRNA stability (32). Furthermore, mutagenesis of just two residues in the C-terminal region of L5 (^{289}Lys/^{282}Arg or ^{289}Lys/^{285}Arg) known to be essential for 5S rRNA binding leads to a lethal phenotype in yeast (25). Previously, our laboratory has shown that *T. brucei* L5 binds 5S rRNA both *in vivo* and *in vitro*, but with a lower affinity (K_d [dissociation constant] = 12 nM) than other eukaryotic L5 proteins (K_d = 2 nM for *Xenopus laevis* L5) (33). The *T. brucei* L5 sequence deviates from

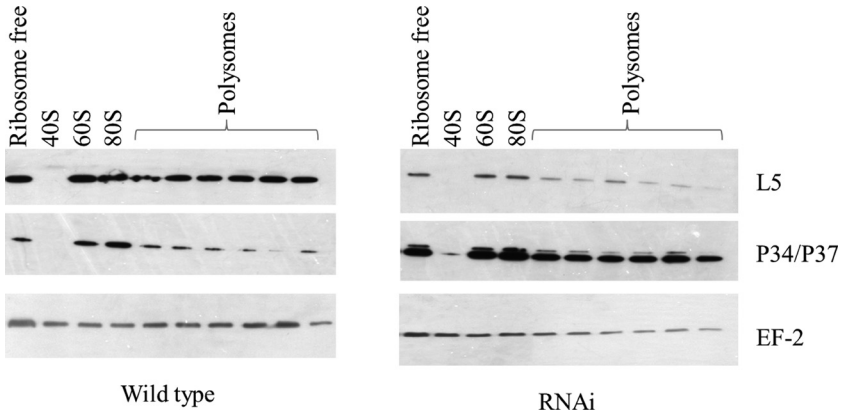

FIG 7 The levels of the P34 and P37 proteins increase in polysomal profile fractions from induced L5 RNAi cells. Fractions from the wild-type and induced extracts were collected, and proteins in the fractions were analyzed by Western blotting using antibodies against L5, P34/P37, and EF-2 as a loading control.

the consensus sequence at key positions, including one at the Arg (position 285 in the yeast numbering, position 291 in the *T. brucei* numbering) in the C-terminal region described above. In addition, only about 25% of cellular 5S rRNA associates with L5 in *T. brucei*, whereas in mammalian cells, nearly 90% of 5S rRNA is bound to L5 (17).

Our laboratory previously identified a pair of 5S rRNA binding proteins, P34 and P37, in *T. brucei* which had been shown to be essential for parasite growth and 5S rRNA stability. Overall protein synthesis decreased significantly in the absence of these proteins with a defect in mature ribosomes (16). These defects are reminiscent of the phenotype of yeast cells lacking L5, but, surprisingly, trypanosome cells lacking P34 and P37 had wild-type levels of L5 (34). We hypothesized that the presence of trypanosome-specific 5S rRNA binding factors P34 and P37 may compensate for a weaker binding between *T. brucei* L5 and 5S rRNA. A logical outcome of this hypothesis is that cells might be resistant to L5 depletion in the presence of P34 and P37. However, as Fig. 1A shows, L5 remains an essential protein in *T. brucei*, even in the presence of alternate essential 5S rRNA binding proteins, P34 and P37. In fact, L5 continues to be essential as the levels of P34 and P37 are increased (as shown in whole-cell extracts [Fig. 2B] and in polysomal fractions [Fig. 7]). However, it is possible that sustained overexpression of P34/P37 in an overexpressor cell line might rescue an L5 RNAi phenotype, and experiments under way in our laboratory will address this hypothesis. The essentiality of L5 in eukaryotes has been demonstrated only in *S. cerevisiae*, where L5 is the only 5S rRNA binding factor required for 5S rRNA stability.

L5 mRNA levels were efficiently depleted in our RNAi cell line upon induction with tetracycline. L5 protein levels were also depleted, although depletion lagged behind mRNA depletion. It has been suggested in other systems, such as HeLa cells, that mRNAs for ribosomal proteins have long half-lives (35). Our results showed that, under our experimental conditions, the level of L5 mRNA is reduced to 9.5% ± 1% relative to that under uninduced conditions by day 1 (Fig. 2A), while the level of the protein is still present at 52% ± 3% (Fig. 2B). This phenomenon is likely due to the relatively short-lived ribosomal protein transcripts (36, 37) but generally stable ribosomal proteins (38).

One of the most interesting results of our study is that in L5-knockdown cells, in addition to the expected decrease of L5, the levels of two other large ribosomal proteins, L3 and L11, were also dramatically reduced. This effect was specific to proteins of the large subunit, as demonstrated by the unchanged levels of small-subunit protein S5 (Fig. 2B). One possible explanation is that the synthesis of these ribosomal proteins is decreased in our induced RNAi cell line. However, our data (Fig. 2A) suggest that this is not the case, since the steady-state mRNAs of L3, L11, and S5 were increased after L5 depletion. Pulse-chase experiments performed in yeast have demonstrated that depletion of an individual ribosomal protein (L16 or rp59) does not alter the rate of synthesis of other ribosomal proteins or rRNAs (39). In addition, it has been shown that excess proteins not incorporated into ribosomes are rapidly degraded (40). In mammalian cells, ribosomal proteins are expressed at high levels beyond those required for the rate of ribosomal biogenesis, and this high level of expression is balanced by the continual degradation of excess unassembled ribosomal proteins in the nucleoplasm (40). Therefore, we hypothesize that the lack of L5 in *T. brucei* leads to an excess of other ribosomal proteins (exemplified by L3 and L11) which cannot be assembled into large ribosomal subunits and are then rapidly degraded. Further studies will address the measurement of half-lives of ribosomal proteins in the absence of L5. Interestingly, knockdown of L5 in *T. brucei* leads to an increase of the levels of P34 and P37 (Fig. 2B). This could be interpreted as a compensatory mechanism, whereby the cell attempts to compensate for the reduced L5 levels by increasing production of a different 5S rRNA binding factor. 5S rRNA has been shown to participate in feedback loops in other organisms involving 5S rRNA binding proteins (such as L5 and TFIIIA in *Xenopus*) (41).

As mentioned above, upon depletion of L5 we observed an increase in the levels of mRNA for several ribosomal proteins, namely, L3, L11, and S5. To our knowledge, no previous reports have linked depletion of a ribosomal protein to an increase in ribosomal protein mRNAs. One possible explanation for this observation involves a feedback mechanism through transcriptional regulation of the ribosomal protein mRNAs. However, regulation of gene expression is predominantly posttranscriptional in trypanosomes. It is more likely, then, that this feedback increases the half-lives of these mRNAs, either by the direct action of stabilizing molecules or by the downregulation of destabilizing factors. These possibilities will be examined in our laboratory in future experiments. The coordination between mRNAs for proteins of the large subunit and those of proteins for the small subunit has been described in other organisms (42, 43). In trypanosomes, where regulation is posttranscriptional, this coordination presumably involves regions in the untranslated regions which are yet to be defined. The extent of the mRNA stabilization exerted by the depletion of L5 remains to be elucidated in further work.

Northern analysis of 5S rRNA shows that L5 is still required for the maintenance of the steady-state levels of 5S rRNA (Fig. 3), despite the presence of P34 and P37. *In vitro* experiments using recombinant proteins showed that both P34 and P37 bind 5S rRNA with a high affinity (48 nM and 40 nM, respectively) and specificity. However, neither P34/P37 (16) nor L5 (this study) is by itself sufficient to stabilize 5S rRNA *in vivo*. We have previously shown that L5 can form a complex with P34, P37, and 5S rRNA, and our present results, combined with our previous data, suggest that the trimolecular complex is necessary in trypanosomes to provide adequate 5S rRNA stability.

In order to investigate whether the effect of the knockdown of L5 was specific to 5S rRNA, we analyzed the levels of other rRNAs. Knockdown of L5 also led to a decrease in the levels of 25S/28S rRNA and 5.8S rRNA, whereas knockdown of P34 and P37 was shown to have an effect only on the stability of 5S rRNA (16). This suggests that P34 and P37 are factors specific to the biogenesis/stability of 5S rRNA, whereas L5 is necessary for proper assembly and stability of the LSU (of which 5S rRNA, 5.8S rRNA, and 25/28S rRNA are components). In the absence of L5, we also observed decreased levels of 60S subunits (Fig. 5A) and instability of mature rRNAs destined for the same subunits (Fig. 3).

The loss of mature large-subunit rRNAs was accompanied by the accumulation of early precursor molecules of 9.2, 5.8, 5.0, 3.9, 3.4, and 0.61 kb (Fig. 4B) which are intermediates in the generation of 25/28S, 18S, and 5.8S rRNAs. The involvement of L5 in the rRNA processing pathway has previously been shown in mammalian and yeast cells (28, 32). The rRNA precursors that accumulate in our cell line are analogous to those that have been observed to accumulate in human cell lines upon depletion of L5, indicating the highly conserved nature of this process. In addition, our results in *T. brucei* confirm that the processes that generate the various rRNAs are highly interdependent (44, 45). It has previously

been shown that depletion of large ribosomal proteins in yeast and human cells leads to an increase of rRNA precursors of both small and large subunits (46, 47). It is worth noting that, against expectations, the levels of mature SSUs are not altered (Fig. 3), while precursors accumulate. Even though we do not currently have an explanation for this phenomenon, this has been previously reported in human cells (47). The role of L5 in rRNA maturation is observed, even though L5 has no known or defined domains (e.g., nuclease or helicase domains) typically required for rRNA processing. It is possible that the presence of L5 associated with the rRNA precursors is necessary for the recruitment of the relevant proteins. In yeast, results from tagging experiments suggest that L5 physically associates with rRNA precursors 35S rRNA (equivalent to the 9.2-kb precursor in our study) and $27SA_2$, $27SA_3$, and 27SB (all of which are equivalent to the 5.8-kb species) (48). This association with early components could account for the fact that several downstream species in both the large- and the small-subunit rRNA pathways are affected by the depletion of L5.

Analysis of ribosomes and polysomes in *T. brucei* L5-knockdown cells showed that the loss of L5 results in a decrease of free 60S ribosomal subunits, which is likely to be responsible for the observed decrease in 80S monosomes and subsequent polysomes and the resulting increase in free 40S ribosomal subunits. Comparable results were obtained in experiments where L5 was depleted in yeast and human cell lines (10, 28). Our finding of decreased steady-state levels of 5S rRNA, 5.8S rRNA, and 25/28S rRNA, which are all components of the LSU, is also consistent with this observation. In contrast, the polysomal profiles obtained from P34/P37 RNAi cell lines (previously reported by our laboratory) exhibit a marked decrease of the 80S peak concomitantly with an increase of the 60S peak, suggesting a role for P34/P37 in subunit joining. Taken together, our current study and our previous data point to distinct roles of these 5S rRNA binding factors involving different steps in ribosomal biogenesis.

To our surprise, we detected maturation intermediates that accumulated in cells lacking L5 in the cytoplasm and found that they cosedimented with ribosomes in polysomal profiles. To date, only our own work in *T. brucei* with the Read laboratory has demonstrated an extended 5.8S rRNA form incorporated in 60S subunits and polysomes when exonuclease XRNE is depleted (49). However, this case involves a 5.8S rRNA variant that is commonly found in the 60S rRNA subunits of other organisms but not in wild-type *T. brucei*.

Under normal conditions, the exosome degrades excess ribosomal precursors (50). The maturation defects caused by the depletion of L5 may produce an excess of unprocessed intermediates large enough to overwhelm the exosome and allow some of these aberrant RNAs to reach the cytoplasm. Another possibility is that L5 and its associated proteins are involved in quality control mechanisms ensuring that only mature ribosomal subunits are exported to the cytoplasm. Improperly processed rRNAs, e.g., those lacking chemical modifications, are normally rapidly degraded in the nucleus since their incorporation into ribosomes can interfere with properly processed ribosomes on the same polysomal chain (51). We have previously reported an association between P34/P37 and the nuclear export components (23). We speculate that L5 may act as a sensor for improperly processed rRNAs and via its contacts with P34/P37 and the export machinery specifically block the nuclear export of improperly processed precursors.

As mentioned above, P34 and P37 are upregulated in whole-cell extracts lacking L5. However, this increased expression is concentrated in the polysomal fractions (Fig. 7). Most of the increase in steady-state P34 and P37 is targeted to actively translating ribosomes. This observation strengthens the suggestion that the link between depleted L5 and overexpressed P34/P37 is functional and perhaps part of a regulatory network since the overexpressed protein specifically localizes to its site of action. In addition, the increased levels of P34/P37 lead to a larger proportion of L5 (already depleted by RNAi) being associated with P34/P37 (Fig. 8).

In summary, we have expanded our studies of the components of a novel complex containing a trypanosome-specific factor (P34/P37), a conserved L5 ribosomal protein, and 5S rRNA. Our detailed analysis presented here has revealed that, even though the overall functions of L5 are well conserved in trypanosomes, some are unique to trypanosomes. More globally, the current study is the first evidence that depletion of a ribosomal protein leads to an increase in the level of steady-state mRNA of another ribosomal protein transcript. It is also the first report to our knowledge of rRNA intermediates accumulating in ribosomes and polysomes as a result of ribosomal protein depletion. In the context of pathogenesis and the ongoing search for effective targets, our work highlights the importance of targeting the specific association between the conserved (L5, 5S rRNA) and nonconserved (P34/P37) components of the preribosomal complex.

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